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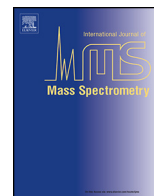
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Electron capture dissociation mass spectrometry of phosphopeptides: Arginine and phosphoserine

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ABSTRACT

We have previously shown that the presence of phosphorylation can inhibit detection of electron capture dissociation (ECD) fragments of doubly charged peptide ions. The presence of non-covalent interactions, in the form of salt-bridges or ionic hydrogen bonds, prevents the separation of fragments following backbone cleavage. Here, we show the electron capture dissociation mass spectrometry of a suite of model peptides designed to investigate the relationship between phosphoserine and arginine position, namely AApSA_nRA_mKA ($n = 0-6$, $m = 6-0$), the presence of lysine residues (AApSAAKAARAKA) and AApSARAAAAKAAAK, and the presence of proline A(A/P)ApSARAAA(A/P)KAAAK. The latter are analogous to the peptides studied previously. The results show that the presence of phosphoserine and basic amino acid residues alone does not inhibit ECD fragmentation, even when the number of basic amino acid residues is greater than the precursor charge state. Neither did the presence of proline in the peptide sequence suppress ECD backbone cleavage. Nevertheless, the presence and relative position of the phosphorylated residue do alter the observed backbone fragmentation abundance. In addition, the presence of phosphorylation appears to inhibit cleavage within the arginine side-chain regardless of the relative position of the arginine residue. The results suggest that ECD fragmentation behaviour is dependent on the three-dimensional structure of a peptide rather than its sequence.

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1. Introduction

Electron capture dissociation is a tandem mass spectrometry technique which has proved highly useful in the analysis of protein post-translational modifications (PTMs) [1]. Capture of electrons by multiply charged peptide ions results in cleavage of N-C α bonds in the peptide backbone producing c and z[•] fragment ions. Importantly for the analysis of PTMs, the backbone fragments tend to retain the modifications [2–6].

Phosphorylation of proteins is one of the most important post-translational modifications and is responsible for numerous cellular processes. Phosphorylation and dephosphorylation are key events in signal transduction, controlling processes including gene expression, cell growth and proliferation. Structural consequences of phosphorylation are variable and dependent of the position of the phosphorylated residue and interactions with neighbouring residues [7–10]. Computational analyses have suggested that a

significant proportion of phosphorylation sites are stabilized via electrostatic interactions with amino acid side chains [11].

Many studies have successfully applied ECD mass spectrometry to the characterization of phosphorylation in peptides and proteins [12–16]. Other work has focused on the effect of phosphorylation on ECD behaviour [17–19]. Turecek and co-workers considered the ECD of doubly-protonated serine phosphorylated pentapeptides containing a single basic amino acid residue (arginine) [20]. The major dissociation channel observed was loss of a hydrogen atom plus phosphoric acid from the charge-reduced species. This unusual fragmentation was attributed to dipole-guided electron capture at the guanidinium side-chain. Previous work in our laboratory revealed that the presence of phosphorylation can have an inhibitory effect on the generation of ECD fragments from doubly-charged peptide ions [17]. Peptides based on the sequence APLSFRGSLPKYSYVK, in which the serine residues were variably phosphorylated, were subjected to ECD and no fragments were detected between the phosphoserine and the arginine residue and/or the lysine residue at position 11. It was concluded that non-covalent interactions between the pSer and Arg and/or Lys were preventing the separation of any fragments formed. More recently, we have used a combination of travelling wave ion

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mobility mass spectrometry and molecular dynamics simulations to probe the structures of those peptides [18]. The results suggest that for the peptide phosphorylated at Ser4 a salt-bridge structure exists, whereas for the peptide phosphorylated at Ser12 ionic hydrogen bonds predominate.

Here, we present the ECD mass spectrometry of doubly charged ions from a suite of model phosphopeptides, see Table 1. The aim was to investigate the relationship between phosphoserine and basic amino acid residues. Pep-01 to Pep-08 were designed to test the effect of the position of the arginine residue in relation to the phosphoserine residue on fragment ion abundance; Pep-09 and Pep-10 were designed to examine the effect of an additional basic residue (lysine); Pep-11 to Pep-18 are analogues of the phosphopeptides studied in our earlier work [17,18] in which all residues with the exception of phosphoserine, proline, lysine and arginine are replaced with alanine. The results suggest that the presence of phosphoserine and basic amino acid residue alone are insufficient to inhibit detection of ECD fragments, even when the number of basic amino acid residues exceeds the charge state of the precursor ion. The inclusion of proline did not result in reduced fragmentation of the phosphopeptides (other than the expected absence of fragments directly N-terminal to the proline residue). The relative abundance of the z^* fragment directly C-terminal to the serine is reduced upon phosphorylation, mirrored by a concomitant increase in abundance of the N-terminal z^* fragment, when arginine is positioned $i \leq +2$. No general trend in abundance of fragments directly N-terminal or C-terminal to arginine and the position of the arginine residue was apparent. For the 12-mer peptides, loss of phosphoric acid was observed in conjunction with loss of ammonia, with the exception of Pep-10 where loss of H_3PO_4 from the charge-reduced radical ion and the charge-reduced hydrogen deficient species was observed. For Pep-12, no loss of phosphoric acid was observed. Loss of CH_5N_3 from the arginine side chain was reduced as a result of phosphorylation but was independent of the position of the arginine relative to the phosphoserine.

2. Experimental methods

2.1. Materials

Model peptides were custom synthesized by GenicBio (Shanghai, China) and used without further purification. Solvents: Ammonium acetate (50 mM) (Sigma-Aldrich St. Louis, Missouri, USA) in water (LC-MS grade, Fisher-Scientific Leicestershire, UK). Methanol (LC-MS grade), water (LC-MS grade), and formic acid (LC-MS grade) were purchased from Fisher-Scientific (Leicestershire, UK).

2.2. Model peptides

1 mg of peptides Pep-01 to Pep-05 was dissolved in 1 ml solution of methanol/water/formic acid (39.9:60:0.1). The peptides Pep-06 to Pep-18 were dissolved in 1:1 methanol/water to a concentration of ~ 1 mg/ml. Stock solutions were diluted in methanol, water, and formic acid (49.5:49.5:1) to a final concentration of ~ 1 μ M. In addition, 1 mg of the crude peptides was dissolved in 1 ml of 50 mM ammonium acetate (pH = 7.5) and further diluted in the same solution to a final concentration of ~ 1 μ M.

2.3. Mass spectrometry

Samples were ionized via nanospray ionization (nESI) in positive ion mode using an Advion Biosciences Triversa electrospray source (Advion Biosciences, Ithaca, NY, USA), at a flow of ~ 200 nL/min. Pep-01 to Pep-12 were analyzed with 7 Tesla LTQ FT Ultra (Thermo Fisher Scientific, Bremen, Germany) and Pep-13 to Pep-18 were

analyzed with a 7 Tesla solariX-XR (Bruker Daltonics, Bremen, Germany). ECD in the LTQ-FT Ultra was performed by isolating the precursor ion of interest in the linear ion trap (automatic gain control (AGC) target 5×10^5 charges, maximum fill time 2 s, isolation width 5 Th). ECD used thermal electrons from an indirectly heated dispenser cathode [21] for 420 ms at “standard” cathode potential of -3.34 V. The precursor ions in the solariX-XR instrument were isolated in the quadrupole and ECD used thermal electrons at a current of 1.50 A, with a cathode bias of 0.6 V and lens potential of 10 V. Pulse length was varied according to each precursor ion between 0.5 and 0.9 s.

Ion mobility experiments of Pep-05, Pep-06, and Pep-07 were performed in a SYNAPT G-2 HDMS mass spectrometer (Waters Corp., Milford, MA, USA) equipped with Triversa electrospray source and a travelling-wave ion mobility cell (TW-IMS) maintained at 3 mbar of nitrogen. TW-IMS was operated at a wave velocity of 350 m/s and wave amplitude of 18.5, 19, and 19.5 V. Collision cross sections (CCS) calibration was performed following the procedure described by Ruotolo et al. [22] and the CCS of the calibration standards were obtained from the reported values by Counterman and Clemmer [23]. Standards for calibration included tryptic digest peptides of BSA, cytochrome c and myoglobin.

Data analysis was performed with Xcalibur software 3.0 (Thermo Fisher Scientific), Data Analysis 4.2 software (Bruker Daltonics), and with in-house software developed at the National University of Colombia. Analyses of Pep-01 to Pep-12 (LTQ FT Ultra) were performed in triplicate. Results from triplicate analyses were used to calculate the standard deviation of the normalized relative intensity (I_i) of each fragment ion (Eq. (1) in supplementary material). p values were calculated to determine the significance between fragment ion relative intensities using the Student's t -test ($n = 3$) at 95% significance level.

3. Results and discussion

3.1. ECD fragmentation patterns

The doubly charged species of the peptides presented in Table 1 were isolated and fragmented by ECD generating low abundance c and z^* fragment ions [24]. In addition, ECD resulted in c^* ($c^* = c - H$), and z fragment ions ($z = z^* + H$) [25]. The fragmentation patterns observed following ECD of the doubly charged peptide ions are summarized in Fig. 1. The ECD mass spectra are shown in Fig. 2 and Supplemental Figures S1–S12. Full fragment ion assignments are given in Supplementary Tables S1–S18. The results show complete sequence coverage for the peptides Pep-01 to Pep-08 regardless of the presence of phosphorylation or site of the basic arginine residue. Similar results were obtained for Pep-09 and Pep-10. Some inhibition of fragmentation was observed in the central region of Pep-12 when compared with its unmodified counterpart (Pep-11). These results are in contrast to our earlier work, in which inhibition of ECD fragmentation in the region between the phosphate group and the basic residues was observed in doubly charged serine phosphorylated peptides with fifteen amino acid residues (e.g. APLpSFRGSLPKSYVK) [17,18]. In that work, we concluded that non-covalent interactions (either salt-bridges or ionic hydrogen bonds) between the phosphate group and the basic amino acid side chains were preventing the separation of any ECD fragments. The results for Pep-01 to Pep-08 suggest that the presence of both phosphate and basic amino acid residue alone is insufficient to result in inhibition of fragmentation, nor does increasing the number of basic amino acid residues over the charge state, as in Pep-10, result in inhibition of fragmentation. Pep-12 (AAA

SARAAAAKAAAK

), which is an analogue of APLpSFRGSLPKSYVK, with the same location of the arginine, lysine and phosphoserine residues, does show

Table 1
Model peptides sequences. Arginine, lysine and (phospho)serine residues are shown in bold.

| Peptide Sequence | Abbreviated name | Monoisotopic mass (Da) | [M+2H] ²⁺ (m/z) |
|------------------|------------------|------------------------|----------------------------|
| AASRAAAAAKA | Pep-01 | 1028.57266 | 515.29361 |
| AApSRAAAAAKA | Pep-02 | 1108.53899 | 555.27677 |
| AApSRAAAAAKA | Pep-03 | 1108.53899 | 555.27677 |
| AApSRAAAAAKA | Pep-04 | 1108.53899 | 555.27677 |
| AApSRAAAAAKA | Pep-05 | 1108.53899 | 555.27677 |
| AApSRAAAAAKA | Pep-06 | 1108.53899 | 555.27677 |
| AApSRAAAAAKA | Pep-07 | 1108.53899 | 555.27677 |
| AApSRAAAAAKA | Pep-08 | 1108.53899 | 555.27677 |
| AASAAKAAKA | Pep-09 | 1085.63051 | 543.82253 |
| AApSAAKAAKA | Pep-10 | 1165.59684 | 583.80569 |
| AAASRAAAAAKAAK | Pep-11 | 1298.74184 | 650.37819 |
| AAApSRAAAAAKAAK | Pep-12 | 1378.70817 | 690.36136 |
| APASRAAAAAKAAK | Pep-13 | 1324.75749 | 663.38602 |
| APApSRAAAAAKAAK | Pep-14 | 1404.72382 | 703.36918 |
| AAASRAAAPKAAK | Pep-15 | 1324.75749 | 663.38602 |
| AAApSRAAAPKAAK | Pep-16 | 1404.72382 | 703.36918 |
| APASRAAAPKAAK | Pep-17 | 1350.77314 | 676.39385 |
| APApSRAAAPKAAK | Pep-18 | 1430.73947 | 716.37701 |



Fig. 1. Summary of fragments observed in the ECD mass spectra obtained for [M+2H]²⁺ ions of peptides Pep-01 to Pep-18. Fragment assignments are shown in Tables S-1 to S-18 of the Supplementary Material.

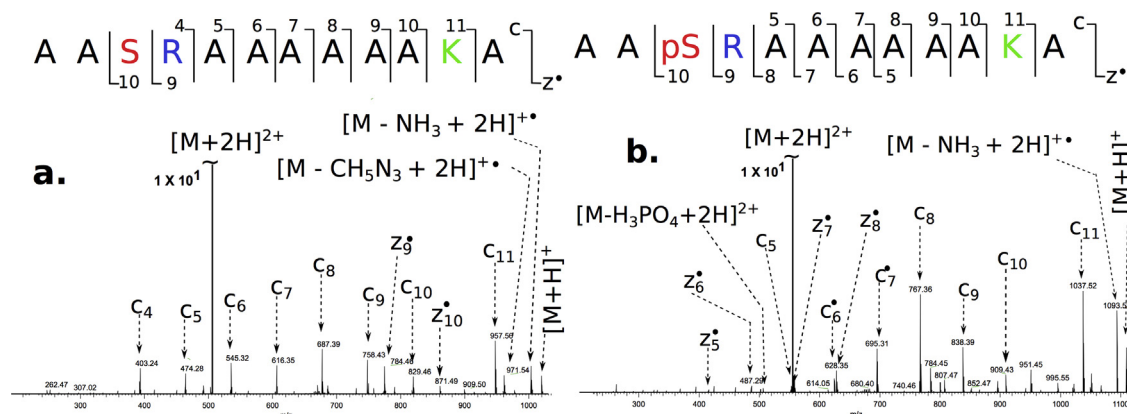


Fig. 2. ECD mass spectrum of [M+2H]²⁺ ions in acidic conditions of: (a) Pep-01; (b) Pep-02. Fragment ion assignments are presented in Supplementary Tables S-1 and S-2.

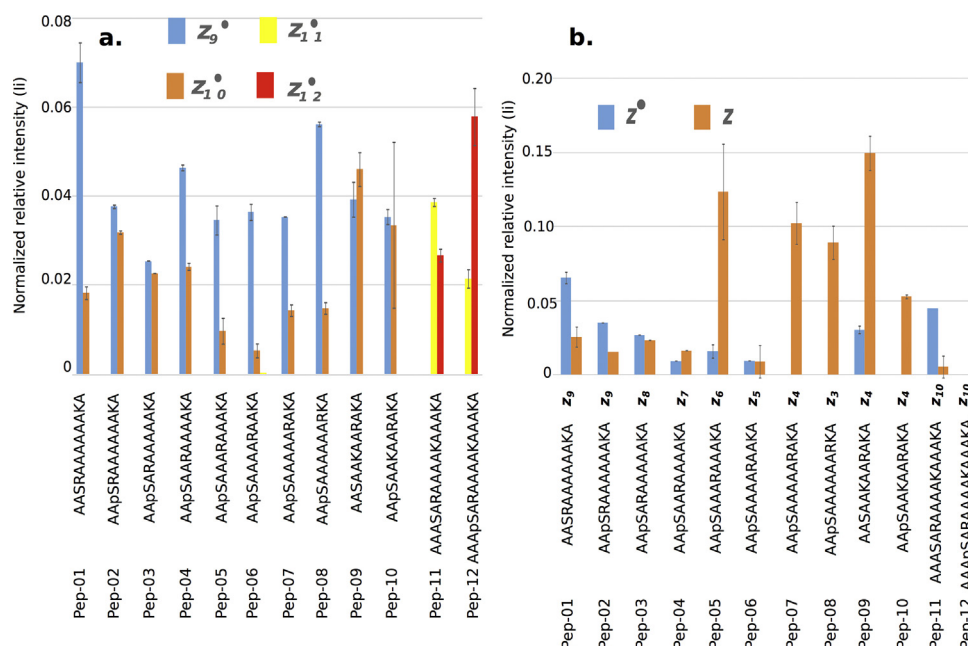


Fig. 3. Results for Pep-01 to Pep-12: (a) Comparison of the normalized relative intensities of the z^* fragment ions adjacent to the phosphoserine residue (...ApSA...); (b) Comparison of the normalized relative intensities of the z^* and z fragment ions N-terminal to the arginine residue. Normalized relative intensities were calculated using equation 1 (see Supplementary Material). The standard deviation of the triplicates is presented in Tables S-29 to S-40, Supplementary Material.

some inhibition of fragmentation but the role of the phosphate and lysine/arginine is not readily apparent. Moreover, inclusion of the proline residue in the phosphorylated peptides Pep-14 (APApSARAAAAKAAK), Pep-16 (AAApSARAAAPKAAK), and Pep-18 (APApSARAAAPKAAK) results only in the usual inhibition at the N-terminal side of the proline residue. (The cyclic nature of proline means that cleavage of two bonds is required in order to observe fragmentation under ECD conditions). We conclude therefore that ECD fragmentation is dependent on the overall structure of the peptide ion rather than simply its sequence. That is, the non-covalent interactions between the phosphate and the basic amino acid side chains responsible for inhibition of fragmentation in the original peptides are promoted by the overall structure.

The ECD spectrum of Pep-01 (AASRAAAAAAKA), in acidic solution, is shown in Fig. 2a. The spectrum shows complete sequence coverage, with c fragment ions in positions 4–11 and z^* radical fragment ions in positions 9, 10 and 11. It is widely accepted that protonation of peptide ions occurs at the basic residues. The residues most likely to accept a proton are Arg > Lys > His > N-terminus. For Pep-01, the predicted protonation sites are Arg and Lys. The fragmentation pattern suggests that, following electron capture, the proton is retained at the guanidine group of the arginine residue and that the charge neutralized as a result of the capture of the electron was located at the lysine residue. Pep-02 differs from Pep-01 in the presence of a phosphorylated serine (denoted as pS, with net mass addition of 79.9663 Da). The ECD mass spectrum of Pep-02 shows the presence of z_n^* radical fragment ions, $n=5$ to 10, abundant c_6^* , c_7^* , and c_8^* (compared to the same fragment ions in Pep-01), and side chain losses (Fig. 2b). Interestingly, the c_4 fragment ion was not observed. The presence of the z_5^* , z_6^* , z_7^* , and z_8^* fragment ions, i.e., fragment ions which do not contain the Arg residue suggests that the structure of the precursor phosphopeptide ion is altered as a result of phosphorylation. Either a zwitterionic structure exists, in which the deprotonated phosphate forms a salt-bridge with protonated arginine and the overall charge of 2+ arises from additional protonation of the lysine residue and N-terminus, or a (non-ionic) interaction between the phosphate and the arginine side chain favours electron capture at

the Arg. It is unlikely, however, that, at the acidic conditions of the experiment, deprotonated phosphate groups are present for this species [20]. Similar fragmentation was observed for Pep-03 (see Figure S-1 and Table S-3, Supplementary Material). In that case, the z_7^* radical fragment ion (which does not contain the Arg residue) was observed. For the remaining peptides containing two basic amino acid residues (Pep-04 to Pep-08), all the backbone ECD fragment ions contained the Arg residues, suggesting an absence of, or weak, interactions between the phosphate and Arg residues. ECD of doubly charged ions of Pep-09 and Pep-10, which contain two lysine residues, one arginine residue and the N-terminus as protonation sites, resulted in backbone fragments, all of which contain the Arg residue. ECD of Pep-12 resulted in formation of a fragment, z_9^* , which did not contain the Arg, unlike its non-phosphorylated counterpart Pep-11. Similarly, fragments z_9^* , z_8^* , and z_7^* , which do not contain Arg, were also present in the ECD spectra of phosphorylated Pep-14, Pep-16, and Pep-18, but not the non-phosphorylated counterparts. These results again suggest interactions between the phosphate and Arg side chain at $i+2$.

To further investigate the above hypotheses, the ECD behaviour of Pep-01 and Pep-02 in ammonium acetate solution at pH 7.5 was determined (Supplemental Tables S-19 and S-20, Supplemental Figures S-14 and S-15). The ECD mass spectrum in native conditions of Pep-02 lacks fragment ions z_5^* , z_6^* , z_7^* , z_8^* , and c_5 (see Figure S-15); whereas that for Pep-01 and Pep-03 remained unchanged (Supplemental Figure S-16 and Table S-21, Supplementary Material). The absence of the z^* radical fragment ions which do not contain the arginine residue (z_5^* , z_6^* , z_7^* , z_8^*) in Pep-02 at higher pH suggests that the conformation of the peptide does not involve interactions between the phosphate and Arg. In other words, z_5^* , z_6^* , z_7^* , z_8^* fragment ions could only be present if non-covalent interactions between phosphate and arginine exist. As deprotonation of the phosphate group is more likely to occur at higher pH, the results suggest that the interactions do not involve salt-bridges. It is well known that charge neutralization at the arginine residue is unlikely to occur by migration of a guanidinium hydrogen to the amide backbone group due to the high transition-state energy required [13,26]; however, it is possible that the phosphate

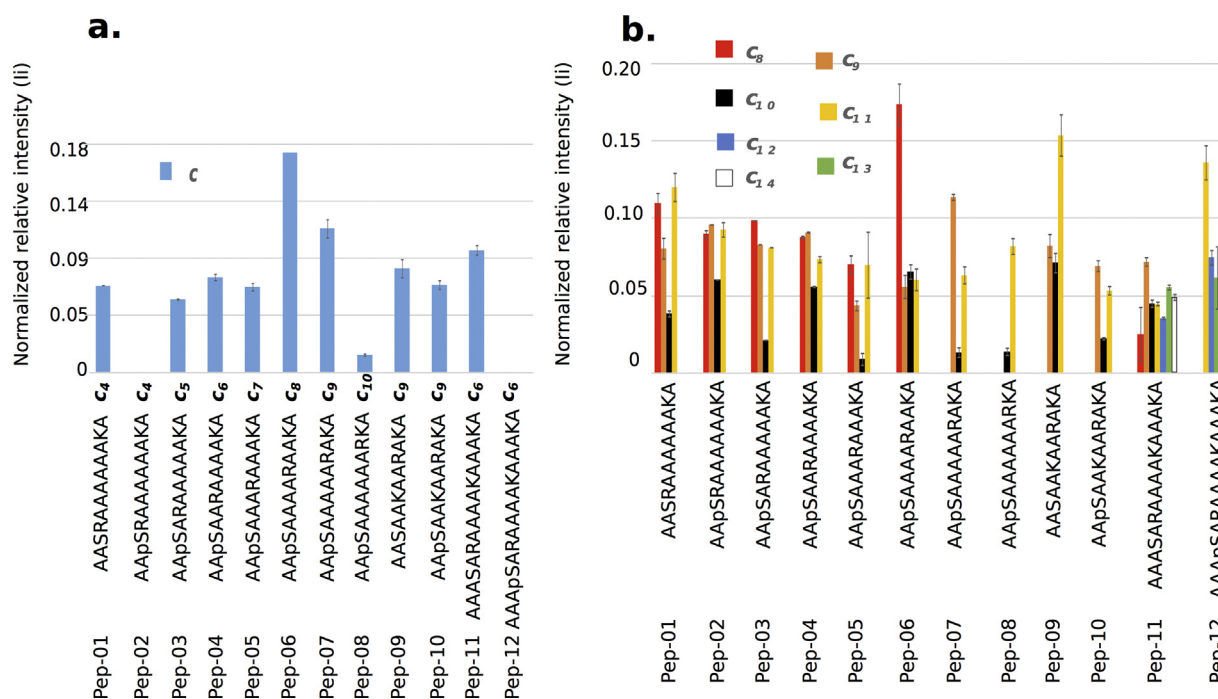


Fig. 4. Results for Pep-01 to Pep-12: (a) Comparison of the normalized relative intensities of the c fragment ions N-terminal to the arginine; (b) Comparison of the normalized relative intensities of the c fragment ions (c_8 to c_{11} (or c_{14})). Normalized relative intensities were calculated using Eq. (1) (see Supplementary Material). The standard deviation of the triplicates is presented in Tables S-29 to S-40, Supplementary Material.

group lowers the energy barrier to donate such a proton or promotes inverse H α migration to the guanidinium group [26,27], leading to backbone cleavage. ECD of Pep-12 at higher pH (Supplemental Table S-22) also resulted in formation of a fragment, z_9^+ (without Arg residue). Similarly, ECD of Pep-14, Pep-16, and Pep-18 at higher pH resulted in z_9^+ , z_8^+ , and z_7^+ fragment ions (without Arg) (Supplemental Figure S-16, Tables S-23 to S-28). Again, these results suggest a peptide conformation in which interactions exist between the phosphate and Arg side chain at $i + 2$.

3.2. Relative intensities of ECD fragment ions adjacent to the phosphoserine and arginine residues

The normalized relative intensities of all fragment ions for the Pep-01 to Pep-12 were calculated by application of equation S-1 (Supplementary Material). See Supplemental Tables S-29-S-40. Here, we focus on fragment ions adjacent to the phosphoserine (...ApSA...), and arginine residue (...AR...), i.e., the z^{\bullet} radical fragment ions N-terminal and C-terminal to the phosphoserine residue, and the z^{\bullet} , z , and c fragment ions N-terminal and C-terminal to the arginine residue. For Pep-01 to Pep-10, the z_9^{\bullet} fragment ion is C-terminal to the phosphoserine residue and z_{10}^{\bullet} is N-terminal, whereas for Pep-11 and Pep-12, the z_{11}^{\bullet} fragment is C-terminal and z_{12}^{\bullet} is N-terminal. The normalized relative intensities of $z_9^{\bullet}/z_{10}^{\bullet}$ or $z_{11}^{\bullet}/z_{12}^{\bullet}$ are plotted in Fig. 3a.

There is a significant decrease ($p=0.01$) in the abundance of the z_9^* fragment from phosphorylated Pep-02 when compared with that for the unmodified Pep-01. Similarly, the abundance of the z_{11}^* radical fragment ion from phosphorylated Pep-12 decreased ($p=0.01$) when compared with the unmodified counterpart (Pep-11). Such a behaviour was previously reported in the ECD of 11-mer phosphorylated peptides [28]. The z_9^* radical fragment ion of Pep-10 does not show a significant decrease in the normalized relative intensity within the error of the measurement ($p=0.16$), which could be attributed to the presence of an additional lysine residue in the fragment ion offering additional stabilization to the radical [29], however, the relative intensity of the fragment ions is

similar to that from Pep-07 which does not contain the lysine residue. The normalized relative intensities of the z_9^+ radical fragment ions from Pep-03 through Pep-08 do not show any correlation with the position of the arginine residue. Thus, it seems that the position of arginine residue does not influence the abundance of the z^+ radical fragment ion C-terminal to the phosphoserine residue.

The normalized relative intensities of the z_1^* radical fragment ions N-terminal to the phosphoserine residue (z_{10}^* for Pep-1 to 10 and z_{12}^* for Pep-11 and Pep-12) are also shown in Fig. 3a. For Pep-02 there is an increase in the normalized relative intensity of the z_{10}^* radical fragment ion compared to that of unmodified Pep-01 ($p=0.002$). Similar behaviour is observed for the z_{12}^* radical fragment ion of Pep-12 ($p=0.03$) compared to Pep-11. For Pep-10, there is no change in the relative intensity of the z_{10}^* radical fragment ion in comparison to that from unmodified Pep-09 ($p=0.49$). Furthermore, the relative intensities of the z_{10}^* radical fragments from Pep-10 and Pep-07, (AAPSAAAAARAKA) do not exhibit a significant difference ($p=0.31$) in abundance that could be attributed to the presence of the additional basic residue (lysine residue). The normalized relative intensity of the z_{10}^* radical fragment ion from Pep-06 (in which the Arg is located four residues away from the phosphoserine) is significantly lower than that from Pep-02 ($p=0.005$).

The normalized relative intensities of the z^* and z fragments N-terminal to the arginine residue are plotted in Fig. 3b. The results show a decrease in the abundance of the z^* radical fragment ion from Pep-02 compared to Pep-01 (as discussed above) and an absence of the z^* fragments from Pep-10 and Pep-12. A decrease in abundance is also observed for the z fragment ions ($z = z^* + H$) from Pep-02 and Pep-10 and an absence from Pep-12. The presence of z fragment ions in ECD spectra are the product of hydrogen migration from the complementary fragment [30], and may not be influenced by the position of the basic residues. The z_5 fragment ion of Pep-06 (Fig. 3b) appears to be an outlier; however a Student's t -test reveals that the z_5 fragment ion abundance is not significantly lower when compared to z_6 of Pep-05 ($p = 0.36$) or when compared to z_4 of Pep-07 ($p = 0.33$). Nevertheless, there is a general increase

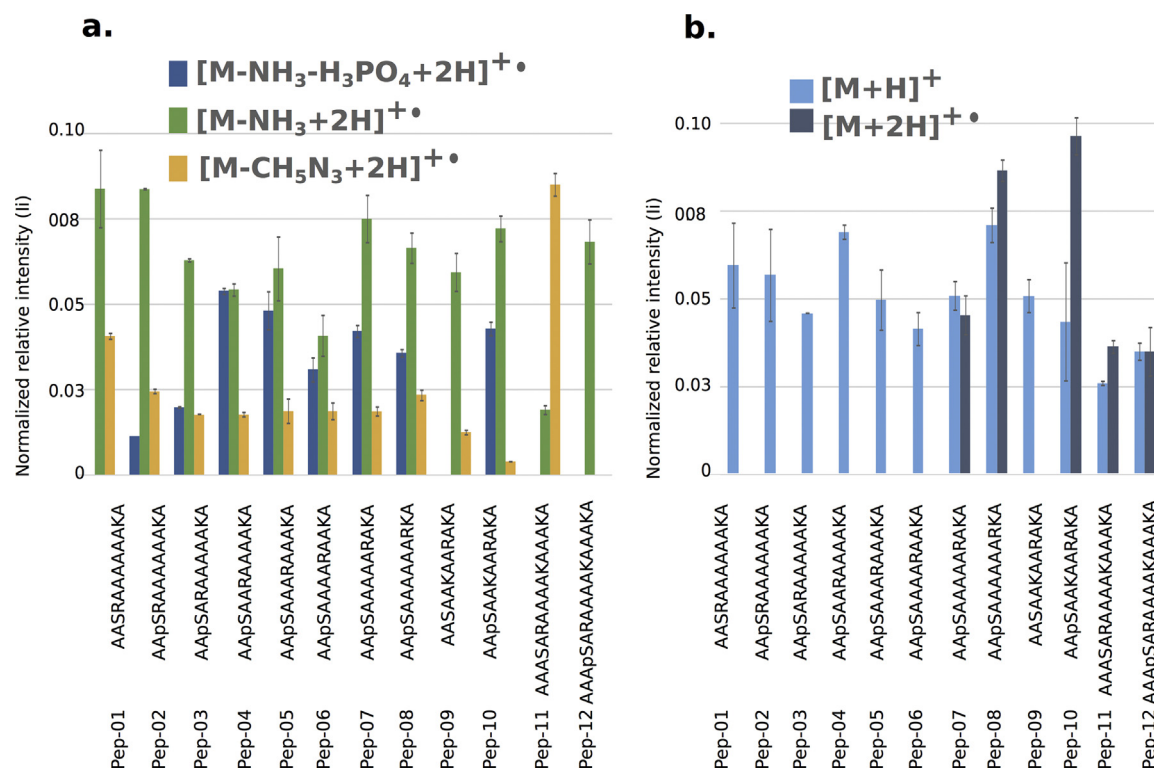


Fig. 5. Comparison of the normalized relative intensities of the side chain losses observed in the ECD mass spectra of Pep-01 through Pep-12. The standard deviation is presented in Tables S-29 to S-40, Supplementary Material.

in abundance of z fragment ions throughout the series of phosphorylated peptides (Figure S-18, Supplementary Material) suggesting that phosphorylation could promote hydrogen migration to the z^* radical fragment ions. No general trend between arginine position relative to phosphate and abundance of z^* radical fragment ions N-terminal to the arginine residue is apparent from Fig. 3b.

The normalized relative intensities of the c fragment ions N-terminal to the arginine residue are shown in Fig. 4a. When the arginine residue is adjacent to the phosphoserine (Pep-02), no c fragment N-terminal to arginine was observed. We speculate that the absence of the c_4 fragment ion in Pep-02 could indicate that there is a non-covalent interaction between the phosphate and the Arg, which renders the c_4 fragment neutral making it undetectable in the mass spectrometer. The c_8 fragment ion from Pep-06 is particularly abundant but similar behaviour is not observed for the c_7 fragment ion of Pep-05, nor the c_6 or c_5 of Pep-04 and Pep-03 (Figure S-19, Supplementary Material). That is, there is no clear correlation between the abundance of the c fragment ions and the position of arginine residue (protonation site) within the sequence. The results suggest that the peptide conformation is dictating ECD behaviour. The significantly high abundance of the c_8 fragment ion from Pep-06 (see also Fig. 4b) ($p=0.0013$ when compared to c_9 fragment ion of Pep-07) suggests that the electron is preferentially transferred to the (Coulomb stabilized) π^* orbital adjacent to the site of capture rather than throughout the peptide, possibly due to distance [31]. The collision cross sections of the $[M+2H]^{2+}$ ions of Pep-05, Pep-06 and Pep-07 in both acidic and native solutions were determined by travelling wave ion mobility spectrometry (Table 2, Supplemental Figure S-20). Comparison of CCS for Pep-05 and Pep-06 suggests that Pep-06 has a more extended conformation possibly indicating increased coulombic repulsion between the protonated Arg and Lys residues. Similar results are seen for Pep-07 vs. Pep-06. These data are inconclusive with respect to the ECD behaviour of Pep-06 and molecular dynamics simulations or ab initio calculations are warranted. Fig. 4b shows the relative intensities

of fragments c_8 through c_{11} (or c_{14}) following ECD of the peptides. The relative intensity of the c_{10} fragment ion from Pep-02 is significantly greater than that of the c_{10} fragment ion from unmodified Pep-01 ($p=0.005$). An increase ($p=0.005$) was also observed for the c_{10} fragment ion of the unmodified species of Pep-07, AAPSAARAARA, compared to that from Pep-10 AAPSAAKAAARA.

3.3. Normalized relative intensities of side-chain loss fragment ions $[M-NH_3-H_3PO_4+2H]^+\bullet$ and $[M-CH_5N_3+2H]^+\bullet$

The ECD mass spectra obtained from the 12-mer peptides (Pep-02 to Pep-08) reveal loss of H_3PO_4 in conjunction with loss of ammonia, but loss of phosphoric acid alone is not observed. The ECD mass spectrum obtained from Pep-10 shows loss of phosphoric acid from both the charge-reduced radical species, $[M+2H]^+\bullet$, and the charge-reduced hydrogen deficient ion, $[M+H]^+$. Moreover, for the 15-mer peptide Pep-12, no loss of phosphoric acid or phosphoric acid plus ammonia was observed (Fig. 5a). This result suggests that the length of the peptide may stabilize the phosphoserine rather than the presence of an additional basic residue (cf Pep-10). Additionally, the ECD mass spectra for Pep-01 to Pep-06 and Pep-09 reveal the presence of $[M+H]^+$, i.e., loss of H^* from the charge-reduced radical species, and absence of the hydrogen abundant radical species $[M+2H]^+\bullet$. It is believed that the loss of ammonia follows the loss of H^* due to the absence of $[M-NH_3+H]^+$ species (Fig. 5b, and Supplementary Figures S-1 to S-4, S-7 and S-11). Both $[M+2H]^+\bullet$ and $[M+H]^+$ species are observed for Pep-07, Pep-08 and Pep-10, which suggests stabilization of the radical species $[M+2H]^+\bullet$ by both proximity of the arginine to the lysine residue and by the presence of an additional basic group (Pep-10). These results differ from previously published results of phosphorylated pentapeptides where the dominant fragments were the loss of a hydrogen atom followed by loss of phosphoric acid [20].

The loss of ammonia is believed to occur from the peptide N-terminus due to the high stability of $C\alpha$ radicals after

Table 2
Peptides collision cross sections (CCS).

| Peptide sequence | Abbreviated name | [M+2H] ²⁺ Acid solution CCS (Å ²) | [M+2H] ²⁺ Native solution CCS (Å ²) |
|------------------|------------------|--|--|
| AApSAAARAANKA | Pep-05 | 267.21 | 265.28 |
| AApSAAARAANKA | Pep-06 | 272.66 | 270.76 |
| AApSAAARAANKA | Pep-07 | 281.91 | 274.54 |

electron capture [20]. Loss of NH₃ from lysine residues is unlikely to occur without the aid of other amino acid residues that favour the formation of a stable radical, as was reported for non-covalent complex of crown-ether and peptides [32,33]. The abundance of the peak corresponding to [M–NH₃+2H]^{•+} is significantly lower for Pep-06 than Pep-05 ($p=0.04$), Pep-07 ($p=0.08$) and Pep-04 ($p=0.0007$), which could indicate that the lysine residue in doubly charged Pep-06 is less well stabilized than in the other peptides, supporting the general conclusion that peptide conformation is central to ECD behaviour. The ECD mass spectra for all the peptides also show loss of CH₅N₃, which is attributed to loss of the side chain from the arginine residue [34]. Arginine residues dissociate by loss of a neutral guanidine moiety rather than by atom migration from the guanidinium group onto the amide carbonyl [35]. The normalized relative intensities of the [M–CH₅N₃+2H]^{•+} species show a decrease upon phosphorylation, see Fig. 5a. We hypothesize that phosphorylation triggers the involvement of a non-covalent interaction that decreases the loss of the side chain from the arginine residue. This observation may be related to the increased abundance of z[•] radical fragment ions N-terminal to the phosphoserine residue, i.e., the z₁₀[•] radical fragment of Pep-02 and z₁₂[•] radical fragment of Pep-12. The increased abundance of these z[•] radical fragment ions could be the result of inverse hydrogen atom migration to the guanidine side chain of the arginine residue, which consequently decreases the abundance of [M–CH₅N₃+2H]^{•+} species. Interestingly, the abundance of the [M–CH₅N₃+2H]^{•+} species remained unaffected by the position of the arginine residue.

4. Conclusions

The doubly charged cations of a suite of model peptides comprising alanine, proline, arginine, lysine and (phospho)serine were subjected to ECD mass spectrometry. Complete cleavage coverage was observed for the phosphorylated peptides with 12 amino acid residues, regardless of whether the sequence contained one or two basic amino acid residues, and inhibition of fragmentation was observed for the 15-mer peptide only. These results suggest that the presence of basic amino acid residues and phosphoserine alone is insufficient to curtail ECD fragmentation. Peptide length and/or other structural motifs may promote non-covalent interactions and thus lack of fragmentation in ECD, i.e., ECD behaviour is dependent on peptide conformation. Additional z[•] radical fragment ions in Pep-02 and Pep-03 indicates the presence of interactions between the phosphate and the arginine residue, but any interaction is too weak to prevent observation of ECD fragments.

The relative intensities of the c/z[•], z, [M–CH₅N₃+2H]^{•+}, and [M–NH₃–H₃PO₄+2H]^{•+}, fragments were normalized and compared within the series of peptides. Increased abundance of the z[•] radical fragment ions located N-terminal to the phosphorylated serine seems to correlate with phosphorylation. A decrease in the normalized relative intensity of the [M–CH₅N₃+2H]^{•+} ion was observed. This observation may be correlated with the increased abundance of z[•] radical fragment ions N-terminal to the phosphoserine residue due to an H_α migration. Enhanced cleavage at the N-terminal phosphoserine residue may suggest that, in the three-dimensional structure of the peptide, the π[•] orbital of the C_α of the serine residue may be located at a distance around 6 Å from the

guanidinium site [31,36,37]; however, computational modelling coupled with ab initio calculations is required to validate this proposal.

Associated content

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijms.2015.07.024>.

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